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Simultaneous measurements of ionic currents and leucine uptake at the amino acid cotransporter KAAT1 expressed in *Xenopus laevis* oocytes

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Abstract

The transport properties of the intestinal amino acid cotransporter KAAT1, heterologously expressed in *Xenopus* oocytes, were studied using simultaneous voltage-clamp and tritiated leucine uptake measurements. While addition of 1 mM leucine to oocytes kept at −80 mV in presence of Na^+ or K^+ caused an increase in holding current, in presence of Li^+ the current was reduced. Uptake measurements in voltage-clamp conditions showed that a comparable accumulation of amino acid occurred in all three ionic conditions and irrespective of the direction and amount of the current change. The ratio of moles of transferred charge to moles of transported amino acid ranges from 1.45 for K^+ to 3.52 for Li^+. A hypothetical interpretation involving the coexistence of two populations of transporters, one operating in the uncoupled mode and the other in the substrate transport mode is discussed.

Keywords: Cotransport; Epithelial transport; Oocyte; Charge movement

1. Introduction

KAAT1 is a neutral amino acid cotransporter that operates in the midgut of the larva of the lepidopteran *Manduca sexta*; its recent cloning [1] has revealed interesting homologies with the superfamily of the Na^+- and Cl^--dependent mammalian neurotransmitter transporters [2]. In addition to the structural similarities, electrophysiological studies on KAAT1, heterologously expressed in *Xenopus* oocytes, have shown that it also shares with this family several functional characteristics, such as a Cl^- dependence [1], and the presence of conspicuous uncoupled and presteady-state currents [3].

An interesting peculiarity of KAAT1 is its ability to use K^+ as driving ion for the amino acid uptake, in agreement with the fact that the diet of the larva is rich in K^+ [4]; however, KAAT1 can also function with Na^+ and with other alkali cations [5].

Studying the ionic selectivity of KAAT1 both in the absence and in the presence of the organic substrate, we have observed [3,6] that among the various alkali cations, lithium gives rise to the largest current when the amino acid is absent, followed by Na^+ and K^+, while in the presence of amino acid, this order is reversed.

These observations are partially in agreement with previous results based on uptake measurements of radiolabeled substrates, which showed that in addi-

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tion to Na^+ and K^+ also Li^+ can support amino acid transport [1,5]. However, the conditions of the electrophysiological experiments are quite different from those of the uptake measurements, as in the former there is no indication of the amount of transported organic substrate and in the latter the membrane voltage is unknown; therefore the two sets of results cannot be easily compared.

In order to directly relate the membrane current with the effective amino acid transport, we have performed experiments in which radiolabeled leucine uptake by KAAT1-expressing *Xenopus* oocytes occurred while keeping the cells at constant voltage and simultaneously measuring the associated currents.

2. Materials and methods

2.1. mRNA preparation

Capped cRNA specific for KAAT1 was synthesized in vitro using *Not*I cleaved pSPORT–KAAT1 cDNA construct with T7 RNA polymerase (Stratagene) [7]. cRNA was phenol–chloroform extracted and ethanol precipitated. The concentration was determined by UV spectrophotometry and integrity was verified by denaturing formaldehyde–agarose gel electrophoresis and by visualization using ethidium bromide fluorescence.

2.2. Oocyte culture and microinjection

Xenopus laevis frogs were anesthetized in MS222 (tricaine methanesulfonate) 0.1% (w/v) solution in tap water, portions of ovary were removed through a small incision on the abdomen, the incision was sutured and the animal was returned to water. The oocytes were treated with collagenase (Sigma Type IA) 1 mg/ml in ND96 Ca^{2+}-free solution (NaCl, 96; KCl, 2; $MgCl_2$, 1; Hepes, 5; all values in mM, pH 7.6), for at least 1 h at 18°C. Healthy-looking oocytes at stages V and VI were collected and injected with 12.5 ng of cRNA in 50 nl of water, using a motorized microinjection system (Drummond, Broomall, PA, USA).

The oocytes were incubated at 18°C for 3–4 days in NDE solution (NaCl, 96; KCl, 2; $CaCl_2$, 1.8; $MgCl_2$, 1; Hepes, 5; all values in mM, pH 7.6 supplemented with 50 μg/ml gentamicin and 2.5 mM Na pyruvate), before electrophysiological studies.

2.3. Electrophysiology and leucine uptake measurements

The classical two-microelectrode technique was used to voltage-clamp single oocytes (Oocyte Clamp, Warner Instr. Corp., Hamden, CT, USA). pClamp 7 software (Axon Instr., Foster City, CA, USA) was used to run the experiments and acquire and analyze the data. When simultaneously performing uptake experiments, the oocytes were placed in small (100 μl) disposable chambers with the desired solution, to which 5 μl of 1 mM L-[4,5-^{3}H]leucine (Amersham) was added. After 5 min of uptake at −80 mV, the oocyte was removed, washed in ND96 solution (see below), dissolved in 200 μl of 10% SDS and radioactivity counted by liquid scintillation. Data from oocytes injected with KAAT1 cRNA were compared with those from non-injected control oocytes.

2.4. Solutions

The external control solution had the following composition (in mM): TMACl, 98; $MgCl_2$, 1; $CaCl_2$, 1.8; Hepes free acid, 5; in the other solutions TMACl was replaced respectively by NaCl, KCl, CsCl and LiCl. The pH was adjusted at 7.6 by adding the corresponding hydroxide for each alkali ion and TMAOH for the TMA^+ solution. Leucine was added at the concentration of 1 mM to the appropriate solutions. The ND96 wash solution had the following composition (mM): NaCl, 96; KCl, 2; $CaCl_2$, 1.8; $MgCl_2$, 1; Hepes, 5; pH 7.6.

Solutions were superfused by gravity onto the oocyte by a pipette tip placed very close (1–2 mm) to the cell.

3. Results

3.1. Uncoupled and coupled currents in presence of K^+, Li^+ and Na^+

In KAAT1-expressing oocytes both the currents in absence or in presence of organic substrates differ,

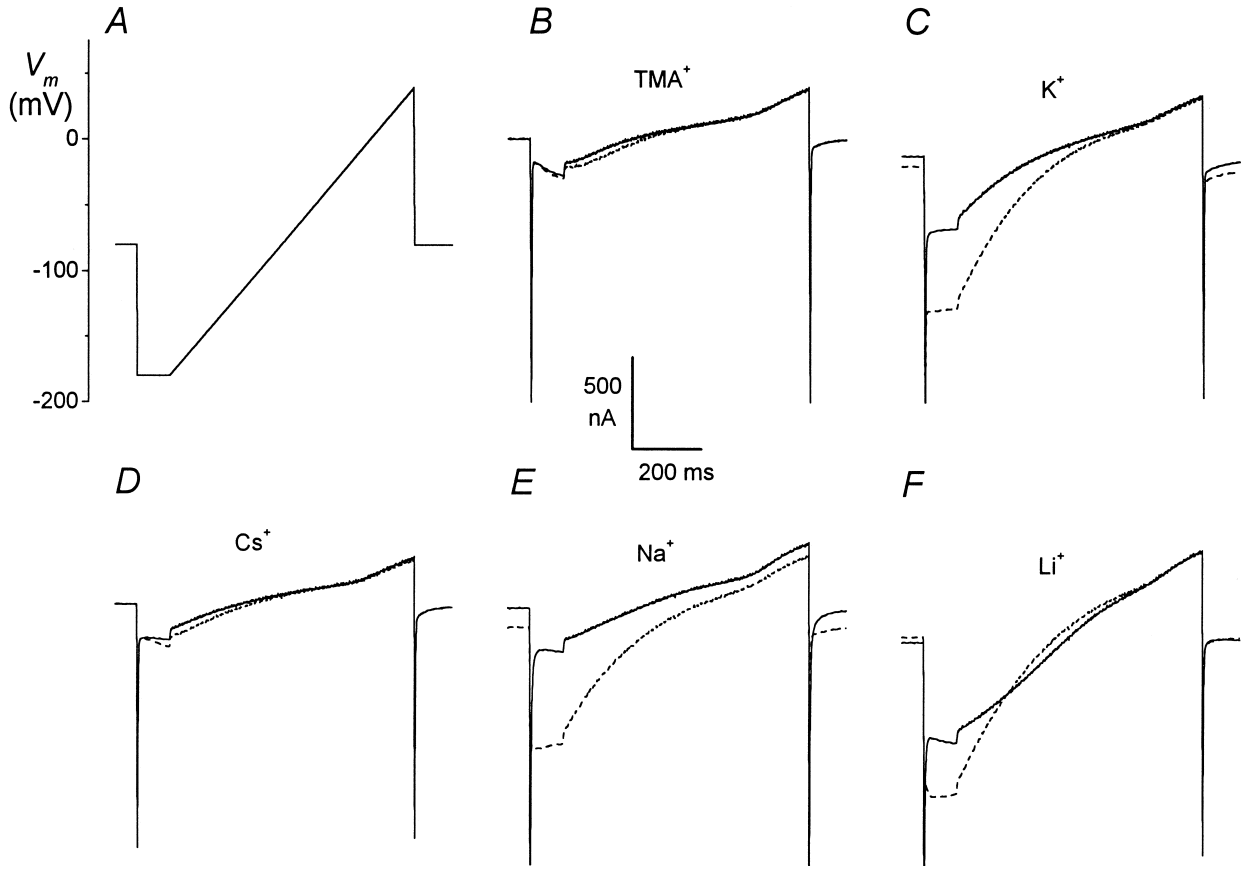

Fig. 1. Currents in the absence (continuous lines) and presence (dotted lines) of 1 mM leucine in KAAT1-expressing oocytes bathed in the indicated ions. The ramp voltage protocol is shown in A. Data from three oocytes of the same batch.

depending on the external cation. This is shown in Fig. 1, where the currents elicited by ramp commands (from −180 to +40 mV, 750 ms duration) in voltage-clamped oocytes are plotted.

It can be seen that the addition of 1 mM leucine modifies only very slightly the current recorded in TMA^+ or Cs^+, whilst in Na^+, K^+ and Li^+ considerable changes, with differing characteristics, can be observed. These can be better appreciated in Fig. 2, where the difference between currents in presence and in absence of leucine is plotted for these three ions as a function of potential. The already noted [1,3] small but persistent Na^+ inward current at positive potentials can be explained by the favorable gradient for leucine; in Li^+, however, there is a region of positive current in the range of potential between −40 and −100 mV (the current values at −80 and −60 mV are significantly different from zero, $P<0.05$). This finding is rather surprising, as it implies that the current in presence of leucine is actually less than the current in its absence. A similar finding has recently been reported for the *Drosophila* serotonin transporter [8].

To better understand this observation it is necessary to assess whether leucine uptake occurs in the voltage range where the decrease in Li^+ current is observed. Lithium has been shown to allow appreciable amino acid uptake in brush border vesicles from *Philosamia cynthia* [4] and modest uptake in *Manduca sexta* vesicles [5]; however, in these experiments the membrane potential was not known.

3.2. Simultaneous coupled current and leucine uptake measurements

In order to directly relate the measured currents with amino acid uptake, we have performed a series of experiments in which the oocytes were kept under voltage-clamp during the incubation in [^{3}H]leucine.

In these experiments the oocytes were voltage-

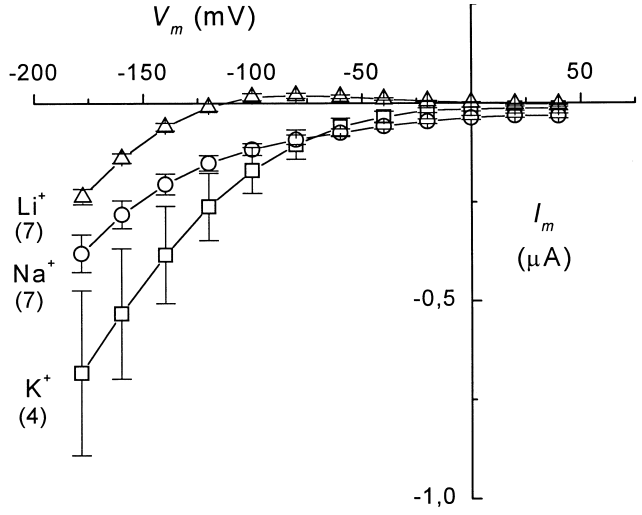

Fig. 2. Difference between the current in the presence and absence of amino acid in the indicated solutions. Data (means ± S.E.) are steady-state currents elicited by 80-ms voltage steps from −180 to +40 mV from a holding potential (V_h) of −80 mV. Number of oocytes in parentheses.

clamped at −80 mV in a solution containing either K^+, Na^+ or Li^+ as the main ion; after a period of stabilization, [^{3}H]leucine was added to the final concentration of 1 mM and the holding current was recorded for the 5 min of uptake period. Fig. 3 shows current traces obtained by averaging a number of recordings in the various conditions.

Table 1
Quantities of transported amino acid and charge at $V_h = -80$ mV

Solution	Leucine (pmol)	Charge (pmol)	Ratio charge/leucine
Potassium	159 ± 46	231 ± 148	1.45
Sodium	150 ± 17	412 ± 57	2.75
Lithium	137 ± 42	483 ± 128	3.52

A slightly declining drift can be seen in the non-injected oocytes, reflecting the usual long-term recovery from microelectrode impalement. In the KAAT1-expressing oocytes substrate addition causes, as expected, a sudden current increase when the bathing ions are Na^+ or K^+, and a small but definite decrease in presence of Li^+. The individual recordings from different oocytes exhibit some variability during the incubation period; however, the time course of the average current shows a slight decline comparable to the controls when the oocytes are bathed in Na^+ or Li^+, while in K^+ a more pronounced inactivation of the current appears.

The parallel leucine uptake measurements are illustrated in Fig. 4A, where the average data from the same oocytes of Fig. 3 are plotted. It can be seen that

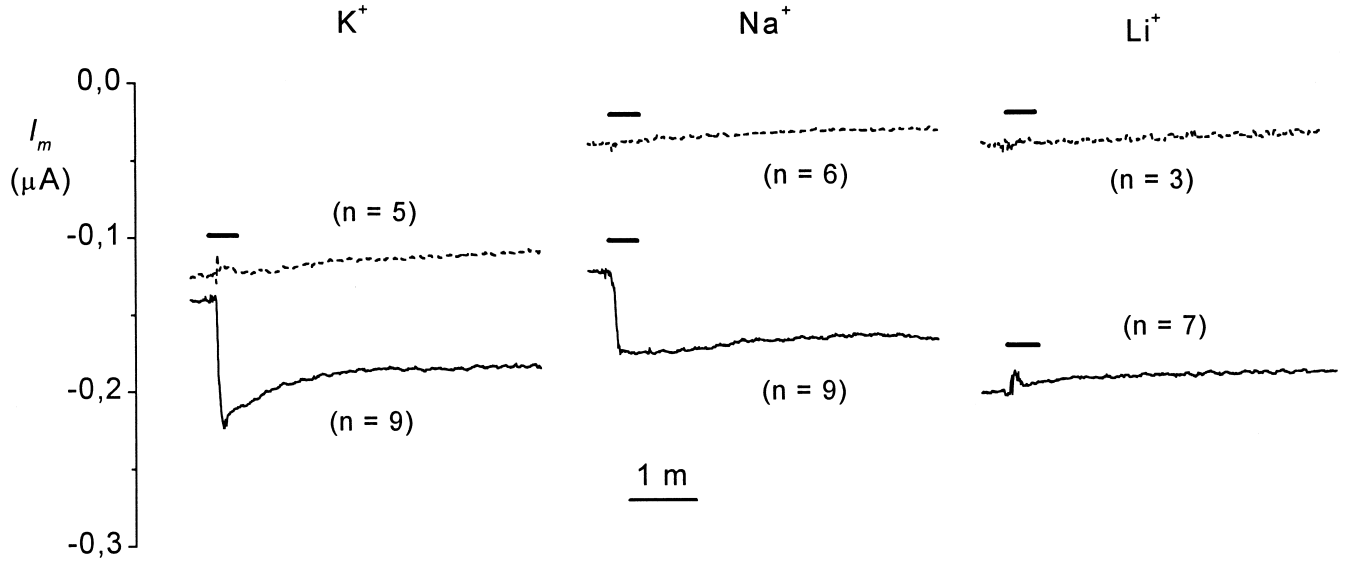

Fig. 3. Average current traces from control (dashed lines) and injected oocytes (solid lines) in K^+, Na^+, and Li^+ solutions, kept at $V_h = -80$ mV during a 5-min uptake period in [^{3}H]leucine. Number of oocytes indicated close to each trace; horizontal bars indicate substrate addition and stirring.

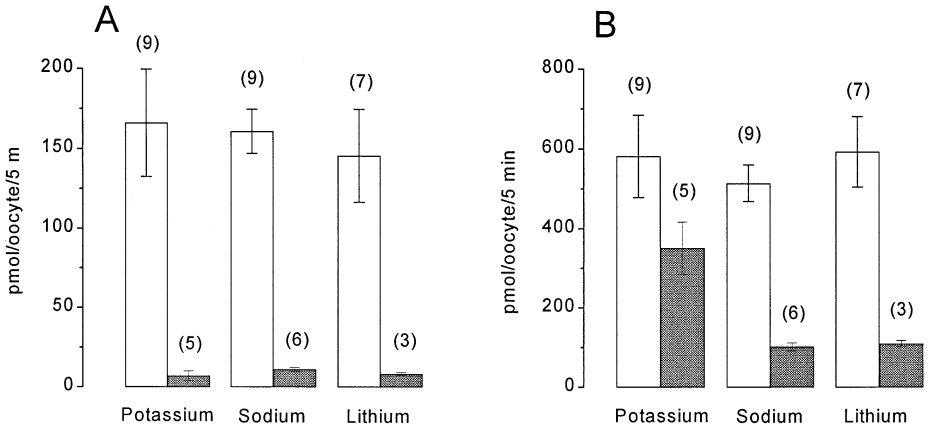

Fig. 4. (A) [^{3}H]Leucine uptake in voltage-clamped oocytes ($V_h = -80$ mV). (B) Corresponding values of the current integrals (converted in picomoles) from the same oocytes. White bars are from injected oocytes, gray bars from controls. Numbers in parentheses represent number of oocytes tested; bars are S.E. of the mean. Oocytes from three different batches.

all three ions support similar amino acid accumulation.

We have also tried to compare the leucine uptake with the amount of charge transferred in these experiments. To this purpose each individual current trace was integrated in the interval corresponding to the uptake period, both for injected and for control oocytes; the mean values of these integrals, converted in number of moles of elementary charge, are shown in Fig. 4B. The relatively large value in control oocytes in K^+ is due to the presence of endogenous channels selective for this ion. The amounts of transported amino acid and of transferred charge in injected oocyte, after subtraction of the corresponding values in control oocytes, are summarized in Table 1 for the three ionic conditions.

The last column of Table 1 indicates a variable number of charges transferred per leucine molecule in the three solutions.

4. Discussion

The possibility of obtaining high levels of expression in heterologous systems has recently allowed the accurate characterization of the electrophysiological properties of several amino acid cotransporters [9,10]. This methodology has allowed to determine that the intestinal amino acid cotransporter KAAT1, cloned from the larva of *Manduca sexta*, shares a number of functional and structural characteristics with the well studied GABA transporter family [1]. Among these characteristics, particularly interesting is the presence of uncoupled currents. The fact that KAAT1 appears to be physiologically driven by both Na^+ and K^+ ions [1,4,5] has prompted us to investigate its properties under various ionic conditions. During this study we have found that the largest uncoupled currents carried by KAAT1 occur in presence of Li^+.

We have observed that, while addition of amino acid to oocytes bathed in K^+ or Na^+ causes an increase of the membrane current, in the presence of Li^+ there is a clear current reduction in the range of potentials -40 to -100 mV (Fig. 2).

In the present work we have performed leucine uptake measurements while keeping the oocyte under voltage-clamp. The holding potential was kept at -80 mV, a value at which leucine causes a current reduction in Li^+ (Fig. 3). The uptake data show that nevertheless a considerable leucine transport occurs in this condition: indeed the uptake is not significantly different from that occurring in presence of K^+ (and Na^+) at the same potential (Fig. 4).

These observations raise the question of the definition of the current 'coupled' to substrate transport. Clearly this current cannot be defined as the difference between the current in presence of substrate minus that in its absence since, in the case of lithium, this current may be outwardly directed while the substrate is entering the cell.

In order to compare the uptake of substrate with

the amount of charge transferred we have then calculated this last quantity as the integral of the current in the presence of substrate minus the current through the endogenous channels in the same ionic conditions. Since these two measurements cannot be done on the same oocyte (due to the lack of a specific KAAT1 blocker), this estimate has been done using the average values of groups of injected and control oocytes belonging to the same batches.

The results, presented in Table 1, indicate a variable charge/amino acid coupling ratio, depending on the cation.

Recently the sugar-evoked current in SGLT1-expressing oocytes was demonstrated to be directly proportional to both Na^+ and αMG uptakes [11]. On the contrary, currents generated by some neurotransmitter cotransporters seem to exceed the values expected for a fixed coupling ratio between ions and organic substrates [12–15].

The existence of an uncoupled mode of operation of the transporter offers the following possible interpretation of our results, which should in any case be taken as speculative. In the presence of substrate there will be two populations of transporters: the first will bind and transport the substrate and ions with a fixed-stoichiometry cycle and with a given turnover rate and the second population will operate in the uncoupled mode, transporting only ions. The two modes of operation will have different turnover rates and each individual transporter will shift between the two modes depending on substrate availability or on other unknown factors. In this view, the increase in current observed upon substrate addition in presence of K^+ and Na^+ and the opposite decrease in Li^+ will require that the turnover rate for the substrate transport mode should be higher than the uncoupled mode when the ion is K^+ or Na^+, but that it should be lower when the ion is Li^+.

The minimum value of 1.45 for the charge/leucine ratio in K^+ (see Table 1) is compatible with a stoichiometry of two cations: 1 Cl−:1 aa (since possibly Cl^- is also transferred by KAAT1 [1]) and with the existence of a population of transporters operating in uncoupled mode. The greater ratios in Na^+ and Li^+ are also consistent with the above hypothesis, considering the higher turnover rate of the uncoupled mode with these ions.

Our findings may also be compatible with a channel mode of transport, as proposed for the 5-HT transporter [13]; indeed KAAT1 shares with the 5-HT transporter of *Drosophila* the peculiar high uncoupled current in Li^+ and its decrease upon addition of the organic substrate [8], although no anomalous mole-fraction behavior has been detected [6].

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References

[1] M. Castagna, C. Shayakul, D. Trotti, V.F. Sacchi, W.R. Harvey, M.A. Hediger, Proc. Natl. Acad. Sci. USA 95 (1998) 5395–5400.
[2] J.N. Cammack, S.V. Rakhilin, E.A. Schwartz, Neuron 13 (1994) 949–960.
[3] E. Bossi, E. Centinaio, M. Castagna, S. Giovannardi, S. Vincenti, V.F. Sacchi, A. Peres, J. Physiol. 515 (1999) 729–742.
[4] B. Giordana, V.F. Sacchi, G.M. Hanozet, Biochim. Biophys. Acta 692 (1982) 81–88.
[5] B.B. Hennigan, M.G. Wolfersberger, R. Parthasarathy, W.R. Harvey, Biochim. Biophys. Acta 1148 (1993) 209–215.
[6] E. Bossi, V.F. Sacchi, A. Peres, Pflugers Arch. 438 (1999) 788–796.
[7] N. Dascal, I. Lotan, E. Karni, A. Gigi, J. Physiol. 450 (1992) 469–490.
[8] C.I. Petersen, L.J. DeFelice, Nat. Neurosci. 2 (7) (1999) 605–610.
[9] M.A. Hediger, M.J. Coady, T.S. Ikeda, E.M. Wright, Nature 330 (1987) 379–381.
[10] H.A. Lester, S. Mager, M.W. Quick, J.L. Corey, Annu. Rev. Pharmacol. Toxicol. 34 (1994) 219–249.
[11] B. Mackenzie, D.D.F. Loo, E.M. Wright, J. Membr. Biol. 162 (1998) 101–106.
[12] A. Galli, R.D. Blakely, L.J. DeFelice, Proc. Natl. Acad. Sci. USA 93 (1996) 8671–8676.
[13] A. Galli, R.D. Blakely, L.J. DeFelice, Proc. Natl. Acad. Sci. USA 95 (1998) 13260–13265.
[14] S. Risso, L.J. DeFelice, R.D. Blakely, J. Physiol. 490 (1996) 691–702.
[15] S. Mager, C. Min, D.J. Henry, C. Chavkin, B.J. Hoffman, N. Davidson, H.A. Lester, Neuron 12 (1994) 845–859.